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Passage through Mitosis Is Required for Oncoretroviruses but Not for the Human Immunodeficiency Virus

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The human immunodeficiency virus productively infects and integrates into cells that have been arrested in the cell cycle with either gamma irradiation or aphidicolin. Integration by oncoretroviruses such as the murine leukemia virus (MuLV), on the other hand, depends on cell proliferation. Although the entire cell cycle is not necessary for MuLV infection, it is essential that the infected cells pass through mitosis. The long terminal repeat circle junction, a marker for nuclear entry, is first observed in MuLV-infected cells immediately after mitosis. These results suggest that mitosis is necessary for nuclear entry of MuLV, but not human immunodeficiency virus, unintegrated proviral DNA.

One way in which the oncovirus subfamily of retroviruses differs from the lentivirus subfamily is in their dependence on host cell proliferation for completion of the viral life cycle. Productive infection by murine and avian oncoretroviruses is prevented in the absence of host cell proliferation (16, 17, 31), and transduction of genes by murine leukemia virus (MuLV)-based retrovirus vectors relies on cell proliferation (24). Lentiviruses such as visna virus and human immunodeficiency virus type 1 (HIV-1) are less restricted by cell proliferation. Visna virus replicates in stationary sheep choroid plexus cells (10) in vitro, and both HIV-1 and visna virus infect terminally differentiated tissue macrophages in naturally occurring infection (22, 26).

Infection of aphidicolin-arrested cells with HIV-1. We previously showed that the ability of HIV-1 to infect nonproliferating cells is not specific to macrophages, because HIV-1 will integrate and produce viral proteins in modified HeLa cells (18) that are arrested at the G_2 stage of the cell cycle (19). HIV-1 mutants that do not make the accessory proteins Vpr, Vpu, Nef, or Vif still successfully infect G_2-arrested cells, as does a HIV-1 viral core pseudotyped with an amphotropic MuLV envelope. MuLV, on the other hand, infects these cells only when they are proliferating.

To eliminate the concern that DNA damage and repair caused by irradiation had an influence on these results, we sought to determine whether HIV-1 could infect cells that had been arrested elsewhere in the cell cycle. Aphidicolin is an inhibitor of DNA polymerase α that either arrests cells in S phase or prevents cells from entering S phase, depending on their stage in the cell cycle when the drug is added (14). Serial DNA content analysis by flow cytometry of HeLa CD4-LTR/β-gal cells (18) treated with aphidicolin over a 3-day course shows primarily a G_1 peak, with a small number of cells arrested in mid-S phase and no cells arrested in G_2 (Fig. 1A). [^{3}H]thymidine incorporation was decreased by 96, 91, and 93%, respectively, on days 1, 2, and 3 in the aphidicolin-treated population of cells compared with the untreated cells.

Arrested or proliferating cells were infected with HIV-1 and assayed for integration and viral protein synthesis as previously described (19). HeLa CD4-LTR/β-gal cells were exposed to aphidicolin at 15 μg/ml for 18 h prior to infection with HIV-1. Cells were maintained in the same aphidicolin concentration throughout the experiment. Proliferating or aphidicolin-arrested HeLa CD4-LTR/β-gal cells (10^6) were infected with HIV-1 stocks at a multiplicity of infection of 1.0 in the presence of 10 μg of DEAE-dextran per ml. HIV-1 gene expression was assayed by immunoprecipitation of newly synthesized [^{35}S]methionine-labeled viral proteins from cells 2 days after infection. The arrested cells produced the major HIV-1 proteins p24, p55, and gp160/120 in quantities similar to those produced by proliferating cells (Fig. 1B).

We also used an "inverse PCR"-based method to determine whether HIV-1 DNA was integrated into the nonproliferating cells. This assay amplifies the cellular DNA which flanks the 5′ end of integrated copies of the virus (19). The 3′ end of the provirus gives a single amplified band because of an internal *Hin*dIII site and therefore serves as a control (19). Since integration is relatively random, there are a large number of different flanking cellular sequences in a population of infected cells. Each different integration event amplifies as a different-sized band. The presence of many different bands in the infected-cell lanes demonstrates the numerous separate integration events that occurred in both proliferating and arrested cells (Fig. 1C). These results show that cell division and passage through S phase are not required for HIV-1 infection. Li et al. (20) recently reported similar results.

LAPSN cannot infect aphidicolin-treated cells. We wished to compare the characteristics of HIV-1 and MuLV infections in nonproliferating cells. The MuLV-based retrovirus vector LAPSN (25) contains the human placental alkaline phosphatase (AP) cDNA (8) inserted into LXSN (23). We used LAPSN as a model for MuLV because the AP marker allows us to count the number of infected cells as soon as 16 h after the addition of virus. This is particularly useful for short-time-course experiments when selection for drug resistance or immunofluorescence staining for viral proteins is impractical. In addition, since the vector is not replication competent, there can be no viral spread from a subpopulation of infected cells.

We used E36 cells, a Chinese hamster lung cell line, for the cell cycle experiments with LAPSN because they are easily synchronized in the cell cycle (11, 12). Synchronized E36 cells were arrested with aphidicolin at the G_1-S border and then infected with various dilutions of LAPSN. In parallel, E36 cells

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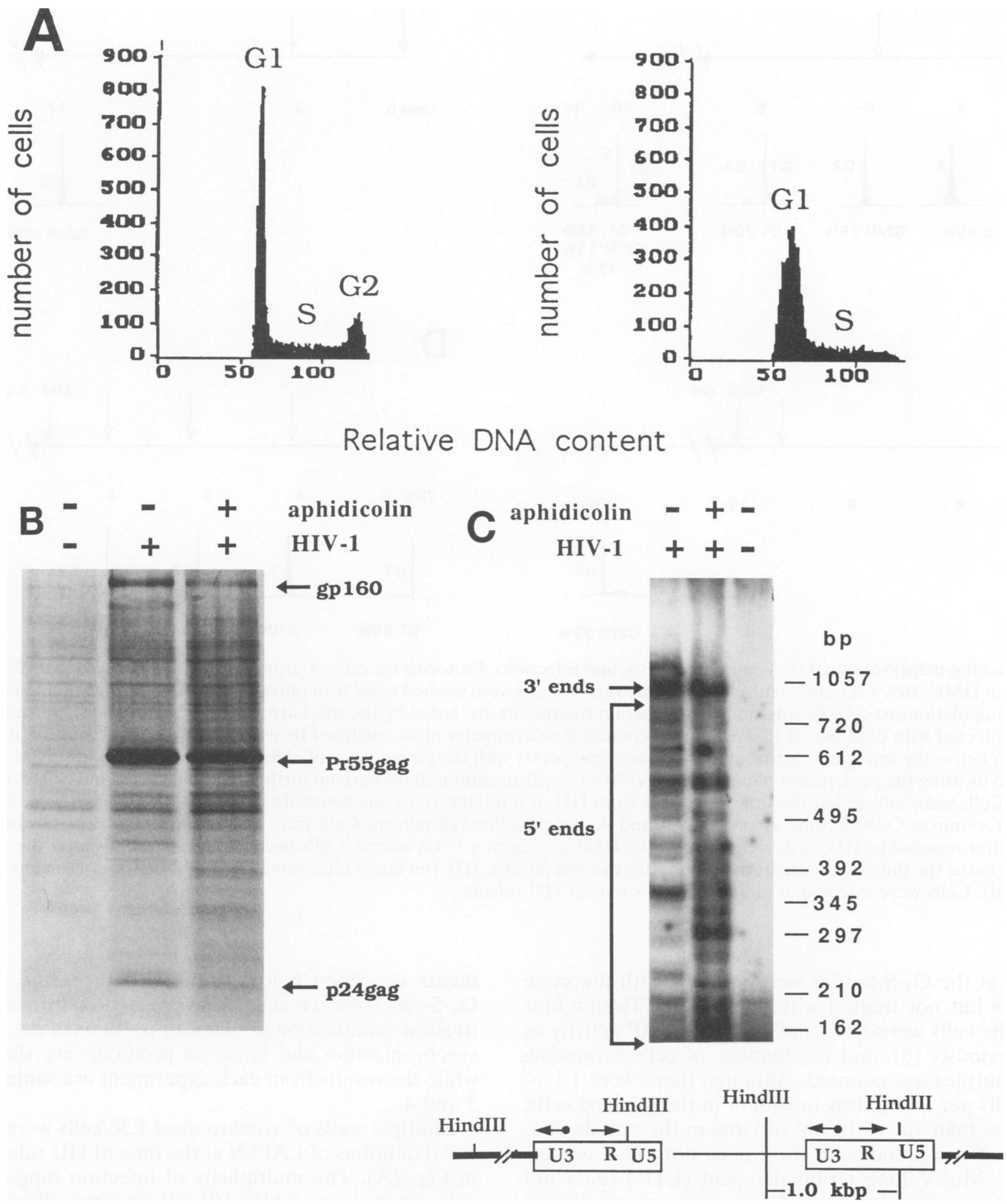

FIG. 1. HIV-1 infection of aphidicolin-arrested HeLa CD4-LTR/β-gal cells. (A) DNA content analysis by flow cytometry of propidium iodide-stained nuclei of HeLa CD4-LTR/β-gal cells (18). Left, growing cells; right, aphidicolin-treated cells after 3 days of treatment (identical plots were obtained after 1, 2, or 3 days). (B) Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis autoradiograph of [^{35}S]methionine-pulse-labeled proteins from HIV-1-infected HeLa CD4-LTR/β-gal cells immunoprecipitated with serum from an AIDS patient. The presence or absence of virus and aphidicolin is indicated above each lane. HIV-1 proteins gp160/120, Pr55^{gag}, and p24^{gag} are noted at the right. Two days after infection with the HIV-1$_{Lai}$ strain (28) at a multiplicity of infection of 1.0, cells were labeled for 1 h with [^{35}S]methionine (250 μCi/ml). The cell pellet was processed for immunoprecipitation with serum from an AIDS patient as previously described (19), size separated on a 12.5% polyacrylamide–SDS gel, and visualized by autoradiography. (C) Inverse PCR analysis of HeLa CD4-LTR/β-gal cells. High-molecular-weight DNA from infected cells (as for panel B) was digested with *Hin*dIII, DNA was diluted to 10 ng/μl and ligated, and 20 ng of DNA was subjected to PCR with primers U3 and R as described previously (19). ^{32}P-labeled primer U3 (antisense) (5′-ATCTTGTCTTCGTTGGGAGTG-3′) and unlabeled primer R (sense) (5′-ATCTTGTCTTCGTTGGGAGAG-3′) were used for PCR. (The sequence of the R primer described in our previous publication [19] was incorrect.) The presence or absence of virus and aphidicolin is indicated above each lane. DNA size markers in base pairs are indicated on the right. PCR products produced from 5′ and 3′ ends are indicated on the left. At bottom a schematic diagram showing integrated HIV-1 provirus and locations of primers used in inverse PCR is shown. The internal *Hin*dIII fragment at the 3′ end of the virus produces the 1-kbp product. The amplified 5′ end will depend on the distance between each integrated provirus and the next *Hin*dIII site.

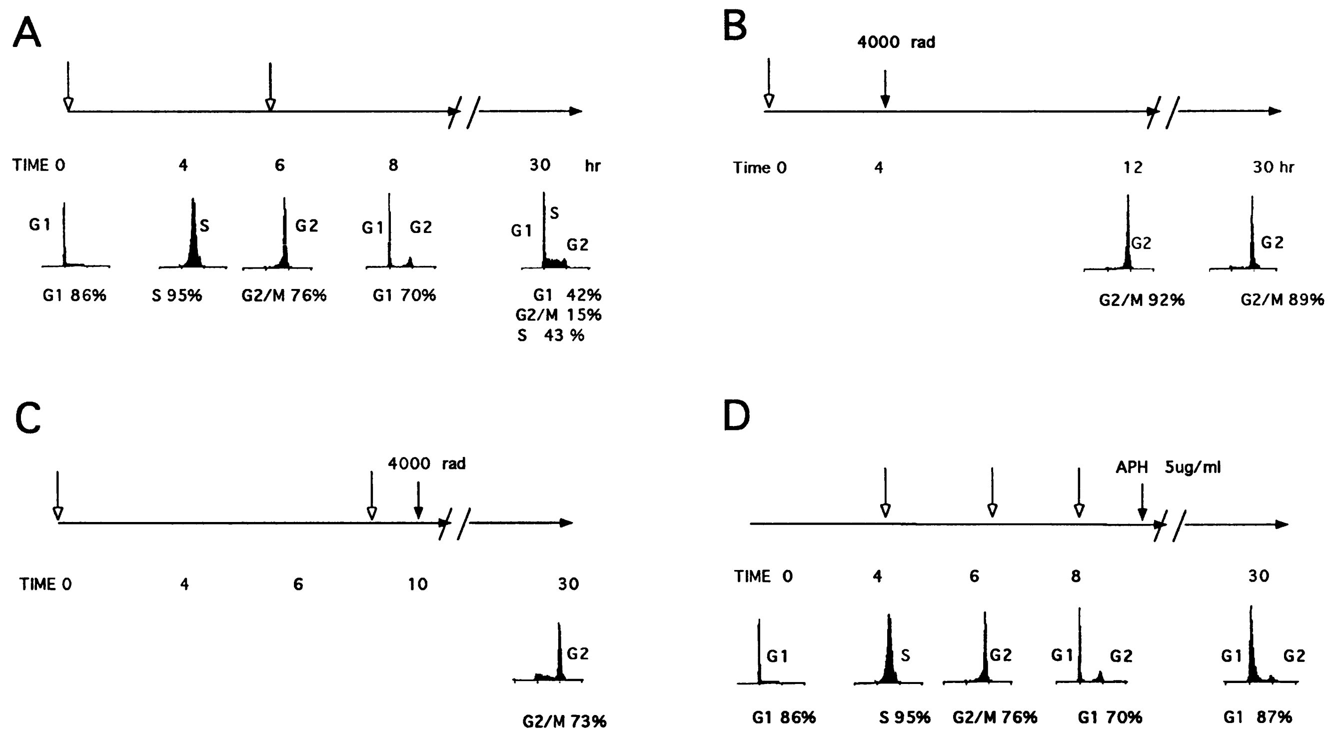

FIG. 2. E36 cell synchronization, DNA content analysis, and infections. Protocols for cell synchronization are all preceded by 24 h in serum-free DME and 16 h in DME–10% CS plus 2.5 mM HU. At time zero all cells were washed twice with phosphate-buffered saline and fed with DME–10% CS. Specific manipulations to cells (irradiation or aphidicolin treatment) are noted by the solid arrowheads. The open arrowheads indicate when the cells were infected with dilutions of LAPSN. DNA content flow cytometry plots, analyzed by using Reproman software (TrueFacts Software Inc.), are shown below the horizontal arrow at appropriate time points with the percentages of cells in either the G_1, S, or G_2-M stage of the cell cycle (estimated by using the peak reflect method [7]). (A) After synchronization there were no further manipulations, and cells eventually become asynchronous. Cells were infected at the time of release from HU or 6 h later. (B) Four hours after removal of HU, cells were exposed to 4,000 rads from a ^{137}Cs source. Cells become arrested in G_2 and do not pass through mitosis. Cells were infected at the time of release from HU. (C) Twelve hours after removal of HU, cells were exposed to 4,000 rads from a ^{137}Cs source. Cells become arrested in G_2 after the second S phase. Cells were infected at the time of release from HU or after the first mitosis. (D) Ten hours after removal of HU, cells were treated with aphidicolin (APH) (5 μg/ml). Cells were infected at either 4, 6, or 8 h after HU release.

synchronized at the G_1-S border were infected with the same virus dilutions but not treated with aphidicolin. Twenty-four hours later, the cells were fixed and stained for AP activity as described previously (8), and the number of cells expressing AP (stained purple) was counted. Although there were 1.1×10^4 purple cells per ml of virus inoculum in the growing cells, there were less than 1.0×10^1/ml of virus in the aphidicolin-arrested cells. Since aphidicolin itself does not affect expression from the MuLV long terminal repeat (LTR) (data not shown), these results indicate that G_1-S arrest prevents infection by a MuLV-based retrovirus vector.

Passage through S phase alone is not sufficient for LAPSN infection. Since proliferating but not arrested cells could be infected with LAPSN, we tested whether cells allowed to pass through less than a full cell cycle could be infected. We used a variety of synchronization and arrest conditions to establish which parts of the cell cycle were required for successful MuLV infection. Cells were synchronized by serum starvation for 24 h and then treated with hydroxyurea (HU) to produce a population of cells arrested at the G_1-S border. The position in the cell cycle was monitored by analysis of cell DNA content by flow cytometry (Fig. 2). When HU-arrested E36 cells were washed free of HU and fed fresh medium, they proceeded synchronously through S phase, G_2, and mitosis (Fig. 2A) and then become increasingly asynchronous. Each of the experiments described below starts with the same population of G_1-S-synchronized cells, which were then further manipulated to allow examination of different portions of the cell cycle. The synchronization and infection protocols are shown in Fig. 2, while the results from each experiment are summarized in Fig. 3 and 4.

Multiple wells of synchronized E36 cells were infected with serial dilutions of LAPSN at the time of HU release (time zero in Fig. 2A). The multiplicity of infection ranged from 0.1 to 0.01. There were 8.4×10^3 AP-positive cells per ml of virus inoculum when the cells were fixed and stained 30 h after infection (Fig. 3, line 1). When cells released from HU were exposed to 4,000 rads from a ^{137}Cs source during mid-S phase, they did not go through mitosis and were arrested in G_2 (Fig. 2B). Although virus was added to these cells at the same time as for the control cells that were allowed to continue through the cell cycle, the cells that became arrested in G_2 (Fig. 2B) were rarely infected by LAPSN (Fig. 3, line 2). These results indicate that passage through S phase is not sufficient for MuLV infection.

As a control, cells were infected at the time of HU release and then allowed to go through mitosis before irradiation. This caused G_2 arrest after the second S phase (see 30-h time point in Fig. 2C). Thus, these cells passed through one entire cell cycle and part of a second. Although none of these cells stained

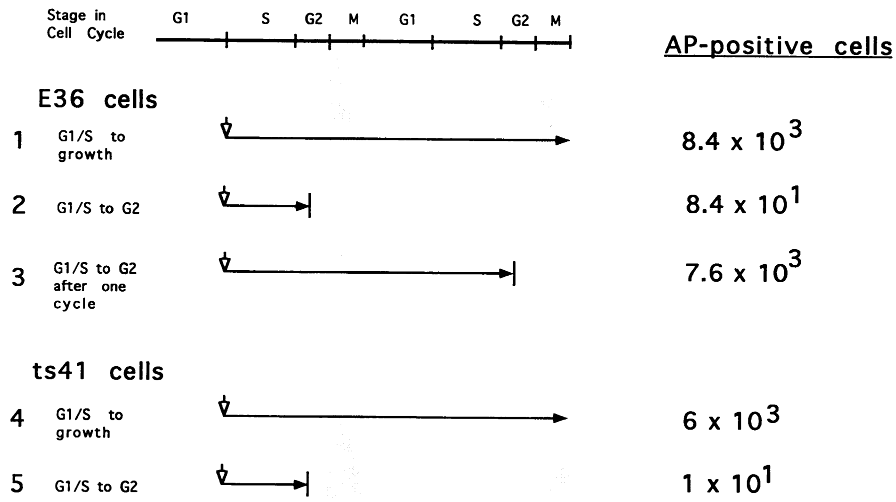

FIG. 3. Number of AP-positive cells in a synchronized cell culture infected with LAPSN when the cells were at the G_1-S border. Limiting dilutions of virus were added to individual wells, and the numbers of stained cells given at the right are normalized per milliliter of virus added. The stages of the cell cycle that the synchronized cells have traversed are summarized schematically on the left with horizontal arrows. Closed arrows ending at a vertical bar indicate cell cycle arrest. A closed arrow not ending at a vertical bar indicates growing cells (lines 1 and 4). Open vertical arrows indicate time of infection. The shaded area represents the time of mitosis. Data from a representative experiment are shown. Lines: 1, E36 cells infected at the G_1-S border and not further manipulated (Fig. 2A); 2, E36 cells infected at the G_1-S border and irradiated 4 h later (Fig. 2B); 3, E36 cells infected at the G_1-S border and irradiated 12 h later (Fig. 2C); 4, *ts*41 cells infected at the G_1-S border and not further manipulated; 5, *ts*41 cells infected at the G_1-S border which become arrested at G_2 because of a temperature shift during G_1 (see text).

positive for AP at the time of irradiation, at 30 h after infection as many irradiated cells produced AP as with cells infected at the same time and not irradiated (Fig. 3; compare lines 1 and 3). This indicates that irradiation, per se, does not interfere with AP expression.

We wished to further test the observation that passage through S phase is not sufficient for MuLV infection by using a cell cycle arrest that did not depend on irradiation. The *ts*41 cell line is a temperature-sensitive mutant of E36 that becomes irreversibly arrested in G_2 if the cells are shifted from 34 to 39°C during G_1 (11, 12). After ts41 cells were presynchronized by serum starvation (G_0), one member of a pair of wells was incubated at 39°C during the subsequent 16 h of hydroxyurea treatment (G_1). Virus was then added to the cells after HU removal, and the cells were allowed to grow for 24 h prior to fixation and staining. The population of cells which had been shifted to 39°C did not progress beyond G_2 (data not shown) and contained less than 1% as many AP-positive cells as the

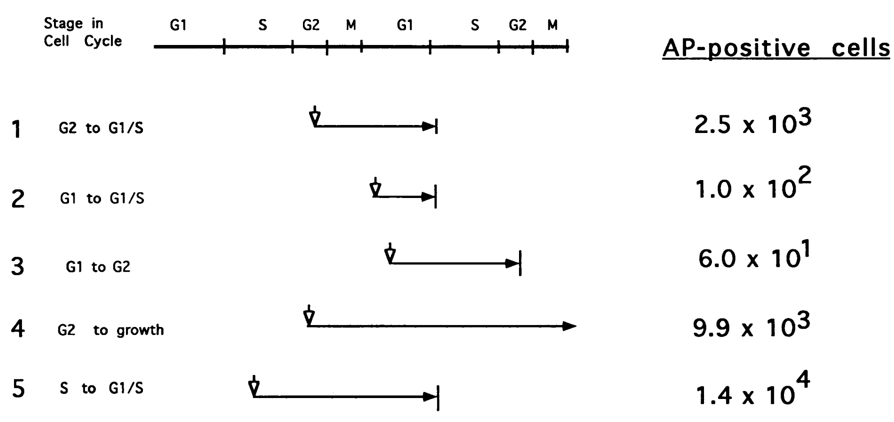

FIG. 4. Number of AP-positive cells in a synchronized cell culture infected with LAPSN at various times after release of cells from arrest at the G_1-S border. Limiting dilutions of virus were added to individual wells, and the numbers of stained cells given at the right are normalized per milliliter of virus added. The stages of cell cycle that the synchronized cells have traversed are summarized schematically on the left with horizontal arrows. Closed arrows ending at a vertical bar indicate cell cycle arrest. A closed arrow not ending at a vertical bar indicates growing cells. Open vertical arrows indicate time of infection. The shaded area represents the time of mitosis. The cells used in this experiment are from the same synchronization experiment as in Fig. 3A. Lines: 1, E36 cells infected 6 h after release from HU and treated with aphidicolin 10 h after release from HU (Fig. 2D); 2, E36 cells infected 8 h after release from HU and treated with aphidicolin 10 h after release from HU (Fig. 2D); 3, E36 cells infected 10 h after release from HU and irradiated 12 h after release from HU (Fig. 2C); 4, E36 cells infected 6 h after release from HU and not further manipulated (Fig. 2A); 5, E36 cells infected 4 h after release from HU and treated with aphidicolin 10 h after release from HU (Fig. 2D).

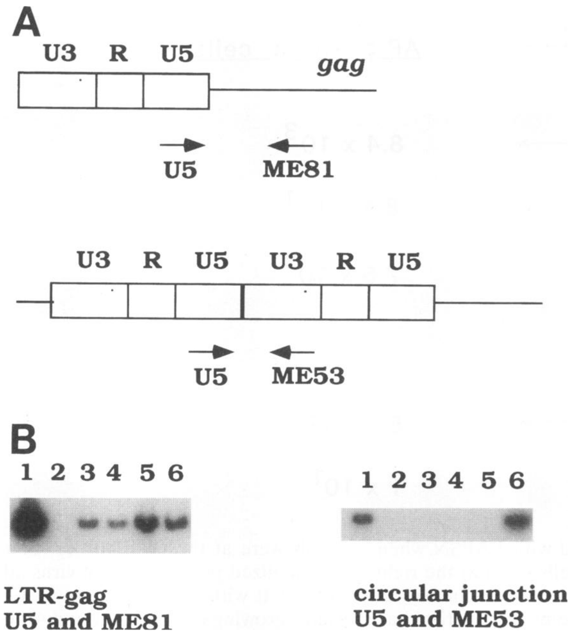

FIG. 5. Circle junctions do not form until the time of mitosis. (A) Schematic diagram of 5′ region of LAPSN, showing locations of PCR primers. Primers U5 and ME81 amplify reverse transcription products that have almost completed (or completed) second-strand synthesis. Primers U5 and ME53 amplify reverse transcriptase products that contain two-LTR circle junctions. Although U5 and ME53 are repeated in the genome, the outside primers (not shown) are too far apart to give a product. Primer sequences were as follows: U5, 5′-CTGAGTGATTGACTACCCACGACGG-3′; ME53, 5′-CTGCTT ACCACAGATATCCTGTTT-3′; ME81, 5′-ACGGGTCCGCCAGA TACAGAGC-3′. PCR mixtures consisted of 1× PCR buffer supplied by Promega; dATP, dCTP, dGTP, and dTTP (all at 0.25 mM); 2.5 mM $MgCl_2$; 0.1 μCi of [α-^{32}P]dCTP; primer oligonucleotides at 1 μM; *Taq* DNA polymerase (Promega) at 1.5 U per reaction; and approximately 10 ng of DNA template. The "hot start" technique was used with paraffin Ampliwax gems (Perkin-Elmer). Temperature cycling consisted of one cycle of 94°C for 4 min; 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; and 1 cycle of 72°C for 5 min. One-fifth of each reaction mixture was size separated on a 7.5% polyacrylamide–Tris-borate-EDTA gel and visualized by autoradiography. The circle junction product was cloned by using the TA cloning system (Invitrogen) and sequenced. (B) PCR analysis of LAPSN-infected-cell DNA by using LTR-*gag* primers U5 and ME 81 or circle junction primers U5 and ME51. Lanes: 1, plasmid positive control; 2, mock-infected cells; 3, DNA from infected cells 2 h after HU release; 4, DNA from infected cells 4 h after HU release; 5, DNA from infected cells 6 h after HU release; 6, DNA from infected cells 8 h after HU release.

population of cells that were maintained at the permissive temperature throughout the experiment (Fig. 3, lines 4 and 5). This confirms the conclusion based on cell cycle arrest with irradiation that passage through S phase and a portion of G_2 is not sufficient for completion of the MuLV retroviral life cycle.

Passage through mitosis is required for infection by LAPSN. Because S phase was not sufficient for MuLV infection, we wished to test whether including mitosis in a partial cell cycle would confer susceptibility. To do so, E36 cells were synchronized at the G_1-S border and then allowed to continue through the cell cycle for 10 h before being treated with aphidicolin (Fig. 2D). At this point over 90% of the cells have gone through mitosis (data not shown), and nearly all become arrested at G_1-S. If virus was added to the synchronized cells 6 h after release from HU, when approximately 76% of the cells were in G_2 (Fig. 2D), infection was surprisingly effective (Fig. 4, line 1). There was only a fourfold reduction in the number of AP-positive cells when virus was added when the majority of the cells were in G_2, compared with cells infected at the same time and not arrested with aphidicolin (Fig. 4, line 4) or compared with cells infected during S phase and subjected to the same aphidicolin block (Fig. 4, line 5). This indicates that a partial cell cycle which includes mitosis and/or G_1 is sufficient for MuLV infection.

Cells were also infected 8 h after release from the HU arrest. At this time point, 70% of the cells had passed through mitosis and most of the population was in G_1 (Fig. 2D). The other 30% of the cells were in mitosis or in late G_2. In contrast to cells infected just 2 h earlier, there were very few cells that could be infected when LAPSN was added at this point (Fig. 4, line 2). This indicates that transduction of the retrovirus vector is not successful if the cells have passed through mitosis before virus is added to the cells.

We also performed an experiment in which LAPSN was added to the culture 10 h after release from HU, when over 90% of the cells are in G_1, and the cells were allowed to progress through the cell cycle until G_2, when they were arrested by irradiation (Fig. 2C). There was no increase in the number of infected cells in this population compared with cells progressing from early G_1 to G_1-S (Fig. 4, line 3). This result shows that even if cells pass through G_1, S, and most of G_2, they cannot be infected with an MuLV retrovirus vector. Cells are susceptible, however, if they pass through mitosis after virus is added.

It had previously been proposed that S phase was necessary for oncoretrovirus DNA synthesis and integration (13, 15, 29). However, here we show that mitosis rather than S phase is the essential cell cycle component needed for oncoretroviral infection. In contrast, HIV-1 can integrate into cells arrested in the cell cycle, and this phenotype is independent of the method used to arrest the cells.

Formation of circular MuLV cDNA at mitosis. We wished to characterize the relationship between mitosis and the synthesis of viral DNA. Because only low multiplicities of infection could be achieved by using the retrovirus vector, we used a PCR assay to detect reverse transcription products. Samples of DNA from infected cells were used as templates in PCR analysis for double-LTR circle junctions by using primers U5 and ME 53 and for complete linear reverse transcripts by using the LTR-*gag* primer pair U5 and ME 81 (Fig. 5A).

Synchronized cells from the experiment in Fig. 2A that had been released from HU and allowed to proceed through the cell cycle were infected with 10^4 infectious units of LAPSN 1 h after release from HU. At 2-h intervals cells were harvested and total DNA was extracted. At 2 and 4 h after release from HU arrest, all of the cells were in S phase (the 4-h time point is shown in Fig. 2A). By 6 h after HU release, most of the cells had completed S phase and were in G_2. By 8 h after release, most of the cells had passed through mitosis and were in G_1 of the next cell cycle (Fig. 2A).

By PCR amplification, reverse transcription products that contained both LTR and *gag* sequences could be detected at all time points after infection (Fig. 5B, left). This DNA results from synthesis across the primer binding site and is a late event in provirus formation. However the two-LTR circular form was not present until 8 h after infection, when the majority of cells have passed through mitosis (Fig. 5B, right). This PCR product was cloned and sequenced and was found to contain an authentic circle junction (data not shown). Therefore, although other reverse transcription products are detected soon after infection, circular DNA is not formed until the cells pass through mitosis.

Although linear, rather than circular, viral DNA is the intermediate to the integrated provirus (2, 21), circular DNA can be used as a marker for entry of viral nucleic acid into the nucleus because circles form only in the nucleus (1, 4). Therefore, the correlation between the appearance of circular viral DNA and the passage of cells through mitosis suggests that viral DNA does not enter the nucleus until mitosis. Indeed, more direct data demonstrating nuclear entry of MuLV cDNA and integration only at the time of mitosis were recently published (30).

Significance of mitosis for retroviral infection. The importance of mitosis in the life cycle of MuLV may be related to the breakdown of the nuclear membrane which occurs at this time or to cell cycle-specific changes in cellular biochemistry. Because it is likely that the MuLV preintegration complex is too large to enter a nuclear pore in the intact nuclear membrane, access to host cell DNA would be prevented until mitosis. It is curious, however, that de novo-synthesized MuLV Pr65^{gag} can be detected in the nucleus (27).

Serum-starved cells infected with oncoretroviruses, in contrast to cell cycle-arrested cells, do not contain full-length reverse transcription products (6, 9). This either may be due to degradation of the viral DNA or may indicate that cells emerging from G_0 must pass through G_1 or S phase before reverse transcription can be completed. This situation may be analogous to the block prior to integration of quiescent peripheral blood lymphocytes to HIV-1 (5, 32).

In contrast to the case with MuLV, nuclear entry of HIV DNA after viral infection does not depend on mitosis (4, 19). Recent work has shown that the HIV-1 matrix protein contains a nuclear localization sequence that can direct a heterologous protein to the nucleus and that mutations in this sequence affect the ability of HIV to grow in cell cycle-arrested cells but not in proliferating cells (3). Further understanding of lentiviral nuclear import may suggest both novel therapies for HIV-infected persons and modifications of current retrovirus vectors for gene therapy to increase the number of susceptible target cells.

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